the pre-coated plates could be kept at least two weeks without loss of their sensitivity (135).

Chandler and Hurrell cited the need for a rapid field test for medical and veterinary use, particularly in developing countries. They noted that the use of horseradish peroxidase system in field situations was limited by its instability and predilection to inactivation by preservatives. They suggested a urease-based system that employed urea as the substrate, liberated ammonia in the presence of a pH indicator, and provided a bright color change that could be easily read. The system could be stabilized with the preservative sodium azide. They devised a field kit capable of testing for the venoms of five Australian snakes. The system was sufficient to detect venom with a sensitivity of 15 ng/ml in 40 minutes. These results compared favorably to their RIA control sensitivity of 10 ng/ml. The kits were successfully field tested, showing a perfect record of identification for the offending snake in 30 minutes of test time (136).

Theakston described the two principal variants in ELISA systems. The double-sandwich, or direct method, is done by coating a 96-well polystyrene plate with antivenin, washing, adding venom, washing, and then adding a specific antibody. The antivenin is conjugated with an enzyme horseradish peroxidase or alkaline phosphatase, the substrate is added, and the hydrolysis observed through a color change. The second variation involves the indirect method in which plates are coated with venom, antivenin is added, and species-specific conjugated antibody applied to label the antigen-antibody complexes. With both techniques it is possible to observe a significant color change (115). The former method would appear to have a greater potential for clinical use.

The first major study on ELISA and North American snake venoms was carried out by Minton et al. in 1984. In this study they employed mice or rats which had been either injected with a

crotalid venom or bitten by the snake. Serosanguineous fluid from the bite site, affected muscle, heart blood and urine were obtained following the death of the animal. The ELISA employed horseradish peroxidase conjugated to antibody (137). The authors found that there were extensive cross reactions with the venoms of some crotalid species so that positive identification of the species, or genus, could not always be made. Venom could be detected in the muscle of mice receiving 0.5 - 1.5 mg of venom, but rarely in their blood or urine. They found that in most cases Crotalus venom could be distinguished from Agkistrodon venom.

The first clinical use of the ELISA in diagnosis of a clinical case in the United States was described by Banner et al. in 1984 (138). In that patient, initial diagnosis was anaphylaxis due to a bee sting. Subsequently, this diagnosis had to be questioned, and the ELISA performed in blood tissue samples, pleural and pericardial fluids, bite site and brain tissues showed that the offending animal was a rattlesnake. The highest ELISA titers were found in brain tissues. Reactions were strongest for Crotalus scutulatus, then Crotalus atrox. In the other body tissues and fluids the strongest reactions were difficult to determine, with perhaps C. scutulatus giving a stronger reaction than C. atrox. There were fewer cross reactions with the four other crotalids tested. Since only C. scutulatus and C. atrox are found in the roadside area where the child was bitten, and the clinical syndrome would seem more typical of a Mojave rattlesnake bite than of the western diamondback, the probable culprit was C. scutulatus.

In his Master's thesis (139), Hitt employed the ELISA to detect and characterize rattlesnake venoms at the species level in samples from rabbit and human sera. Antibodies were prepared separately in rabbits against the venoms of 11 crotalids and an elapid. Sera were taken at different intervals post-immuniza-

tion. In some studies, the prepared rabbit sera were used, while in others an affinity column product was employed (140). Four antibody and three venom dilutions were studied. There was a consistently high correlation in the results for both the antivenin and the venom dilutions when species-specific products were used. This was less obvious in the cross-reaction studies. In these latter studies, C. s. scutulatus antivenin appeared to give the greatest cross reactions, and Agkistrodon piscivorus the least of the six venoms studied. It is interesting to note the close correlation of these findings with the data presented by Gingrich and Hohenadel (141) on the neutralization of the lethal property of the venom.

Hitt found that the horseradish peroxidase system appeared to be the most practical clinically, but felt that if some of the problems he experienced with the urease system could be remedied, this technique might prove more accurate. Subsequently, Hitt and Russell were unable to detect significant differences between the venoms of the subspecies C. viridis helleri and C. viridis oreganus, nor between A. contortrix contortrix and A. contortrix laticinctus, using the horseradish peroxidase ELISA in 40 venom samples (142). These various studies would seem to indicate that, at least with the North American crotalid venoms, one should be able to distinguish between specific species, provided species-specific venoms and antivenins are compared for the snake in question and then compared with those of related snakes. The differences, however, would usually be quantitative rather than qualitative.

This brings up the question of the value of the test in clinical medicine. In those areas of the world where several genera or even families of venomous snakes abound, such as in Africa, Southeast Asia and Australia, there is little question as to the clinical importance of the ELISA. In the United States, however, it is evident that the quantitative differences

at the species level are difficult to evaluate unless species-specific antibodies are used; and as there are fewer than 20 species of rattlesnakes (Crotalus and Sistrurus), one species each of the water moccasin and copperhead (Agkistrodon), and only two genera of coral snakes (Micrurus and Micruroides), and the clinical syndromes are sufficiently different that few physicians became confused, the test would seem of minor clinical value. Also, in this writer's experience, at least 80% of the victims in the United States see the offending snake and generally can identify it. Furthermore, except in certain areas of the country, overlapping does not present a serious differential diagnostic problem. Lastly, since there is only one commercial antivenin for crotalids in the United States, and since this appears to be effective against the venoms of all North American species, a positive identification at the species level is not imperative for good therapeutic care, even though it would be supportive.

In an unpublished paper presented at a recent symposium, Minton reported that in clinical cases, wound aspirates, serum and urine are the most suitable materials for venom detection (143). He appears to have reached a conclusion similar to that presented by Hitt. Minton's ELISA's were done on human tissue samples sent to him by various physicians throughout the United States. He concludes:

> Current ELISA systems involving snake venoms have rather low specificity. Most cannot readily differentiate venoms of related snakes. Venom antibody detection assays are less satisfactory than those for venom. Non-specific reactions and cross-reactivity are unacceptably high (143).

Perhaps this is reflected by the fact that while both Minton and Hitt can be considered as experts on ELISA techniques, in several of the samples that this writer sent to both, different results were obtained. One might attribute this to changes that could have occurred during shipment of the samples, but this fac-

tor has not yet been determined. Suffice to say, this writer agrees with Hitt and Minton that, with respect to crotalids, the ELISA for crotalid venoms in the United States is of limited value as a clinical tool as the assay now stands.

Historically, the ELISA and CELIA have gradually replaced other methods for detecting and quantitating substances with toxinological properties. The high degree of sensitivity of these assays is due to their properties of specificity, high affinity, reversibility of the binding of antigen to antibody, and to the existence of methods for attaching sensitive, detectable labels (isotopes, free radicals, bacteriophages, etc.) to the antigen or antibody.

The principal objective in preparing an enzyme-labeled antigen or antibody is to obtain a stable conjugate with a high titer of immunoreactivity and enzyme activity. The sensitivity of the assay depends on the binding constant of the antibody and on the specific activity of the labeled immunoreactant. It is well known, however, that the linkage of an enzyme to an antigen or antibody may affect the specificity of an assay if the chemical modification alters or masks key immunological determinants. This may account for some of the discrepancies found in the literature with respect to snake venom ELISA studies.

While the ELISA has a number of advantages over the RIA, the complications of quantitating a label as large as an enzyme in the complex, as compared to an atom of iodine, have not been thoroughly studied immunochemically.

G. Antigenic Relationships

It is well known that there are certain antigenic substances in snake venom that appear to be common to several if not many of these toxins. Based on the work of Bordet and subsequently Nuttall and others, a precipitating substance was first observed by Lamb in 1902 (109). He noted a precipitation cross-reaction

with Vipera russelli venom when using a Naja naja antiserum. Two years later he studied several more venoms and found weak cross-reactions between cobra antivenin and the venoms of the sea snake (Enhydrina schistosa) and the green tree viper Trimeresurus gramineus) (67). He pointed out that the neutralizing property of the antivenin bore no relationship to the precipitation content of the serum, a fact that still has applicability.

Hunter confirmed the presence of common antigens in cobra and Russell's viper serum and venom (144). Flexner and Noguchi (66) also demonstrated that "there is no relation between the degree of protection afforded by and the amount of precipitation present in immune serum." They demonstrated a lack of protection between a Crotalus antiserum and the venoms of the cobra and Russell's viper.

Githins and Butz found that the antiserum for Crotalus atrox neutralized the venoms of five other North American rattlesnake venoms (145). It was less effective against Agkistrodon venoms, and of little value against two South American pit vipers. Subsequently, Githins and Wolff, on the basis of these and other studies, divided North American crotalids into three antigenic groups. The first group contained those snakes producing delayed neurological manifestations, the second, all other Crotalus species, and the third, the Agkistrodon (146). Picado stated that there was a "neurotoxin" in the venom of the Costa Rican arboreal vipers (Bothrops) that is not antigenically homologous with Crotalus durissus "neurotoxin" but which is neutralized by the Bothrops antisera (147). Some years later, Akatsuka demonstrated that antivenin against the habu gave weak reactions with the venoms of a cobra and a pit viper (148).

Kellaway demonstrated that animals immunized against tiger snake venom were not protected against the venoms of the Australian brown snake, black snake, or death adder, although they were resistant to the venoms of the Australian copperhead

and taipan (149). Taylor and Mallick found that antivenin prepared against the cobra and Russell's viper reduced the hemorrhagic activity of the saw-scaled and European vipers (150). Ahuja found that puff adder antivenin neutralized the hemorrhagic activity of Russell's, European, and saw-scaled viper venoms (151). Grasset and Schaafsma found that the venom of the poisonous colubrid Dispholidus typus could not be neutralized by any of five elapid antivenins, but antivenins for an African viper and a South American pit viper gave slight protection (152). A more complete review of recent works on antigenic relationships will be found in the various works of Minton, publications of the proceedings of the International Society on Toxinology, and various issues of Toxicon.

It has also been known since the fine study of Lamb that some venom antigens are common to both a snake's blood and its venom. In some cases, there are stronger reactions between antigens and the snake's serum than between the venom and the antiserum to that venom. Some of these antigens have even been found in the blood of nonvenomous snakes, particularly Natrix and Elaphe. The anticomplement protein in Naja naja venom has been reported to be an altered form of C3 of cobra venom (108).

There is no doubt that there is a definite relationship between morphology and taxonomy on one hand and common antigens on the other. The Elapidae and the Viperidae share some common antigens. The Viperidae and the Crotalidae share many, while the Elapidae and the Crotalidae share few.

The venom of one of the so-called more primitive vipers gives precipitation lines with a number of elapid and viperid antisera, as well as with the venomous colubrid Dispholidus. There is also a very weak cross-reaction between Dispholidus venom and a number of pit viper venoms, as well as that of at least one elapid, Pseudechis papuanus. The carpet viper, Echis carinatus, has several antigens in common with the cobra Naja

nigricollis, and one of these is shared with other species of Naja, Hemachatus and Walterinnesia. Naja haje and N. nigricollis antivenins neutralize moderate amounts of Echis and Cerastes venoms (82).

When one comes to study antigenic relationships at the species and subspecies levels, there appear to be many similarities and fewer differences than at the genus level. In fact, the antigenic differences at the subspecies level may not be evident even with our present-day technology, if such differences exist at all. In comparing the venoms of C. viridis helleri and C. v. oreganus over the 200 miles in which they border or coexist, we could find no antigenic differences in the venoms taken from ten adult specimens of each subspecies of snake captured during July and held for one month prior to milking. Minor individual differences could be demonstrated on recirculating isoelectric focusing, but these were not specific for either subspecies. An antivenin prepared against the former gave equal protection in mice against the latter (153). Minton, however, has obtained distinctly different immunoelectrophoretic patterns with two morphologically similar horned vipers, Pseudocerastes p. persicus and P. p. fieldi (82).

These similarities and differences bring up the question as to the definitiveness of morphological characteristics as the sole determinant on which taxonomy can be based. Antigenic relationships, serum and venom proteins, and the use of the ELISA or similar tests may eventually provide us with a more consistent tool for establishing speciation.

Another problem arises when applying immunodiffusion or immunoelectrophoretic data to the clinical problem. While these tests give us information on antigenic similarities and differences, it must be remembered that they do not necessarily identify those venom components responsible for the deleterious action of the venom, if single components are indeed implicated.

A further question that sometimes arises in relation to the significance of reptile venom antigenic relationships was observed during the preparation of antivenin in goats (154). It was found that in order to produce the exact hemorrhagic effect as that evoked by the crude venom, at least three venom fractions identified by antigen-antibody reactions needed to be employed: one that alters the intimal lining of the capillary wall, the second affects the integrity of the red blood cell membrane, and a third which accentuates these alterations. Indeed, these three components gave about 90% of the crude venom's hemorrhagic activity.

Interestingly enough, one can follow this mechanism of action in the clinical case (155). Early on in most patients it becomes obvious from the swelling and edema following rattlesnake envenomation that excessive amounts of fluid are accumulating in the subcutaneous tissues. It is also obvious that this fluid, for the most part, is coming from the blood. This finding is reflected by the early hemoconcentration found in most patients with moderate to severe Crotalus venom poisoning. One may also find an increase in electrolytes in the fluids of the involved tissues, and this can again be seen by a fall in serum electrolytes. In the more serious poisonings, these phenomena are often followed by the frank loss of blood into the affected tissues and by a fall in hemoglobin and hematocrit values. It is possible that in another venom, even one closely related, that one or two of these components might be recognized by ELISA or other assay techniques, but the third might be missing even though, unless closely quantitated, the reaction might be present.

III. GENERAL PRINCIPLES OF ANTIVENIN PRODUCTION

Most present-day antivenins are refined concentrates of equine serum globulins prepared in a liquid or dried form. These are obtained from horses that have been immunized against a

venom, or a number of venoms. Antivenins have now been prepared for use in the treatment of most types of snake venom poisoning. Their effectiveness in neutralizing both the deleterious and seemingly non-deleterious effects of a specific venom may vary considerably, and depends upon a number of factors. The most important of these factors are the specificity of the antivenin, the titer of antibodies, and the degree of concentration or purification of the final product.

In general, the more specific an antivenin the greater the likelihood that it will neutralize the challenging venom. Some fractions of one venom, however, may be common to a number of venoms from a given genus of snakes, and indeed even from that particular family of snakes, and thus an antivenin prepared from the venom of a single species may protect against the venoms of other snakes. Monovalent antivenins are usually the preparation of choice when the offending snake is known, and a monovalent antivenin is available. The various works of Boquet (74-78) and Minton (79-83) indicate some of the important relationships for monovalent and polyvalent antisera.

Polyvalent antivenins have the advantage of not only mitigating the effects of those venoms used in the immunizing mixture but, as in the case of the monovalent antisera, a number of other related venoms. Antivenin [Crotalidae] Polyvalent (Wyeth), an antitoxin prepared with the venoms of four crotalids (Crotalus atrox, C. admanteus, C. durissus terrificus and Bothrops atrox), neutralizes the venoms for 17 species of the family Crotalidae, including the toxins of some Agkistrodon (156). In clinical practice, this antivenin is used in the treatment of poisoning by some 65 Crotalidae in North, South, and Central America, and has been employed in the treatment of bites by certain Asian species of Trimeresurus and Agkistrodon. This should not be interpreted to imply that this antivenin is equally effective in all poisonings by Crotalidae. There is considerable variation in the

neutralization titer of this antivenin for some crotalid venoms, as there is for any polyvalent antivenin.

When the species of the offending snake is not known or is in doubt, polyvalent antivenins play a particularly important role. Another advantage of polyvalent sera is that in many areas of the world where many species or even genera of snakes abound, the disposition and packing of a single polyvalent antivenin is much easier than carrying several or many different kinds of antivenins, and since it is often necessary to carry or store more than a single vial of each antivenin, this presents a physical as well as a financial hardship.

The venoms used in the immunizing mixture are generally obtained from a reliable source, or more often provided by the immunizing laboratory itself. In earlier days, the suppliers of these venoms were not always careful about the control of their product, nor were the samples taken from a broad enough spectrum of snakes to be representative of the species. Further, there was a tendency to use only adult snakes, which of course provide more venom than juvenile or small snakes, and with less chance of mishap during the milking procedure.

It has been demonstrated that the venom of snakes of the same species from different geographical locations may possess both quantitative and qualitative differences, and that the venom of juvenile snakes may be different from that of adult snakes of the same species. Thus, it becomes obvious that the immunizing mixture may not always represent the venom possessed by the offending snake.

This problem was addressed by the Food and Drug Administration of the United States in the early 1960's and by the WHO in 1967, but even today ideal or representative mixtures of immunizing venoms have not received the attention they deserve. It might be said that the efficacy of an antivenin more often reflects the nature of the venom sample than the nature of the

snake venom poisoning, as it exists clinically.

Another shortcoming has involved the testing or assaying of antivenins. Up until only a few years ago the only test employed to determine the efficacy of an antivenin was neutralization of the LD_{50}. Today, some producers are employing and evaluating tests for other neutralizing activities, including hemolysis, and the neurotoxic, myotoxic, and other properties. These procedures should result in a more comprehensive understanding of the properties of antivenins, and hopefully better antivenins. In spite of the shortcomings, however, most antivenins are effective against the venom(s) for which they are produced (157). Improved techniques such as the RIA and ELISA should lead the way to the production of much improved antisera during the next decade.

Another problem that has troubled both laboratory and clinical toxinologists has involved the technique for assaying the antivenin ED_{50}. Based on the fine work of Fraser in 1895, the common method for determining the efficacy of an antivenin is to mix a given amount of the material with a given amount of venom, let the mixture stand for 30 minutes, and then inject it into mice, thus determining the neutralizing capacity of the antisera against the LD_{50}, LD_{99}, or some other lethal parameters of the venom. Obviously this has no clinical relationship, but while it lacks that association, the technique is the most widely employed and accepted method of testing antivenins at the present time. This writer has found, as did Fraser and others, that the length of time the two solutions are incubated is a very important factor in the neutralizing capacity of the antivenin. In fact, it may be the most important parameter that is commonly overlooked by the experimenter. In determining the effective titer of an antivenin using this technique, it is imperative that each mouse test reflect doses of exactly the same amounts and volumes, and exactly the same durations of incubation.

One last general consideration relates to attempts to "beef up" antivenins. At the WHO meeting in 1967, A. de Vries and F. E. Russell reported on their attempts to prepare an improved antivenin by adding certain chemically isolated deleterious fractions of venoms to the crude immunizing mixture. These antivenins were termed "hyperimmunized antivenins", and while they showed promise experimentally, only in Israel were they produced commercially.

These studies might be viewed as the forerunners of present monoclonal or polyclonal techniques now being employed in the experimental production of antivenins. Whether or not the raising of polyclonal antibodies for venom fractions will prove to be more effective than previous attempts with hyperimmunized antivenins has yet to be demonstrated. It is certainly hoped that it will be.

Antivenins employed in clinical medicine have been prepared in horses, donkeys, cattle, goats, and rabbits and other kinds of animals for laboratory or experimental use (1). Horses are by far the most frequently employed host, not only because of their size (an average 2,000-pound horse has 60 liters of blood), but because of their longevity, ease of care, competitive cost, high yield of antibodies, and finally their seeming ability to withstand greater insult from the injections of snake venom. In our production of antivenin in goats during the 1970's, we found that the overall cost of producing 100 grams of antivenin in goats was more than twice that for horses.

Most horses used in an immunization program are healthy animals of 1,500 to 2,000 pounds which have been observed and acclimatized for several weeks, and demonstrated to be free of equine diseases, particularly brucellosis and glanders; usually, they have been treated for equine diseases particular to that area prior to immunization.

The venom to be used in the immunizing program is obtained from healthy snakes and generally from the larger individuals of

the species. It would be hoped that the venom would also be collected at different times of the year and from snakes representing the entire distribution area, but as previously noted, this is rarely done. The venom is then desiccated, dried, or lyophilized. In some cases this is done immediately following milking of the snakes (which is preferred), while in some facilities the venom is stored in the refrigerator or frozen and lyophilized when a certain amount is attained. Recent studies indicate that some enzymes may be denatured while the venom is in liquid form even though its lethal property may not be affected.

The venom is then added to a common pool which may vary from 100 grams to several kilograms, from which samples are routinely taken for control studies, and from which venom is taken for the inoculation of the horse. The venom is subjected to some form of steriliation for contaminating bacteria. In most cases this involves incubation with 0.5% formalin for 24-30 hours at 37°C. Irradiation or other methods of sterilization are sometimes used.

In preparing a monovalent antivenin, the neutralizing capacity of the antiserum is checked against a specific venom. In preparing a polyvalent antivenin, several or many venoms are pooled and injected, and following test runs the neutralizing capacity of the resulting antisera are studied against the venoms in the immunizing mixture as well as against other related medically important venoms. Sometimes the battery of testing includes the venoms of snakes seemingly unrelated to the group under study. In the case of the Wyeth antivenin in the United States, during the formative years of testing (1947-1954) a number of investigations involving various combinations of venoms were made. According to Criley (156) at least five different combinations of venoms were studied for their neutralization capacity of the venoms of 11 American snakes. He observed a

strong relationship between the venoms of Crotalus and Bothrops, and subsequently noted the importance of using those venoms having increased proteolytic and neurotropic properties (156). After extensive testing between 1952-1954, he suggested that the polyvalent antivenin for American Crotalidae should be prepared from four venoms: Crotalus atrox, C. adamanteus, C. durissus terrificus, and Agkistrodon piscivorus.

The venom(s) is put into a saline or saline phosphate buffer solution to make a concentration of 1-2%, then mixed with an adjuvant to retard absorption. The kind and amount of adjuvant varies with the producer's experience. The South African Institute of Medical Research, which has had long experience in antivenin production, employs bentonite as an adjuvant (157). Others use Freund's adjuvant, while Wyeth Laboratories uses aluminum hydroxide gel in a 10% concentration. The formula for the mixture of the venoms to be used in the inoculations varies considerably with each producer. In Wyeth's present program, the venoms are mixed in the following proportions:

Crotalus adamanteus	25%
Crotalus atrox	25%
Crotalus d. terrificus	33%
Bothrops atrox	17%

The venoms are then sterilized, usually by incubating the mixture at 37° with 0.5% concentration of formalin for 24 to 30 hours. The current hyperimmunizing schedule at Wyeth Laboratories is shown in Table I. The adjuvant is 10% Amphojel.

In reviewing eight immunizing programs from different laboratories, it would appear that most facilities inject measured amounts of the immunizing mixture into horses over a period of 10-20 weeks. This is usually followed by a rest period of 3-5 weeks, and then a maintenance schedule is introduced in which the horse is given approximately one-third the immunizing dose, and one week later twice that, and one week after that

TABLE 1
Current Hyperimmunizing Schedule

Weeks	Dose (mg)	Saline 0.85% (ml)	AMPHOJEL Vol (ml)	Dose (ml)	Gram % Venom	Injection Route	Sites
1	3	2.7	0.3	3	0.1	subcutaneous	1
2	6	5.4	0.6	6	0.1	subcutaneous	1
3	10	9.0	1.0	10	0.1	subcutaneous	1
4	24	22.6	2.4	24	0.1	subcutaneous	1
5	50	45.0	5.0	50	0.1	subcutaneous	2
6	100	90.0	10.0	100	0.1	subcutaneous	4 (1 for each venom)
7	200	18.0	2.0	20	1.0	subcutaneous	4 " " " "
8	400	36.0	4.0	40	1.0	subcutaneous	4 " " " "
9	600	54.0	6.0	60	1.0	subcutaneous	4 " " " "
10	750	67.5	7.5	75	1.0	subcutaneous	4 " " " "
11	1000	90.0	10.0	100	1.0	subcutaneous	4 " " " "
12	Rest 9 days, bleed for serum and test for potency.						
13	Rest 5 days.						
14	800	72.0	8.0	80	1.0	subcutaneous	4 " " " "
15	1200	108.0	12.0	120	1.0	subcutaneous	4 " " " "
16	Rest 9 days, bleed for serum.						
17	Rest 2-3 days, bleed for serum.						
	Rest 1 month.						
	Maintenance Schedule						
22	500	45.0	5.0	50	1.0	subcutaneous	4 (1 for each venom)
23	750	67.5	7.5	75	1.0	subcutaneous	4 " " " "
24	1200	108.0	12.0	120	1.0	subcutaneous	4 " " " "
	Rest 9 days, bleed for serum.						
	Rest 2-3 days, bleed for serum.						
	Rest 1 month and repeat.						

(F. J. McCarthy, Personal Communication, 1987)

Current hyperimmunizing schedule used by Wyeth Laboratories for the production of Antivenin [Crotalidae] Polyvalent.

approximately the full dose. In most cases the venom mixture is given subcutaneously, and generally in two to five different areas. The time of bleeding the horses and the amounts vary considerably with the different producers. Some horses have been bled over 70 times during the 15 years they were used for immunization. In general, three to eight liters of blood are taken from a horse at each bleeding. The red blood cells from each bleeding are injected back into the horse at the next bleeding.

The amounts of venom mixture given with each injection over the 10- to 20-week course varies with the specific venom or venoms being given, with the response of the horse, and with the experience of the producer. In the horses we immunized against Crotalus viridis helleri venom in the late 1960's, we gave 5 mg in the first injection and 1,000 mg in the last injection 15 weeks later. Our maintenance injections were 300 mg, 600 mg, and 1,000 mg after a one-month rest period. We bled the animal at 16 weeks and again at 10-day intervals between the 22nd and 38th weeks.

The blood is collected, the serum purified, and the product processed for packaging. The essentials of these procedures are given by the WHO (158). The ideal of the WHO is that an antivenin "should be an almost pure $F(ab)_2$ plasma fraction. The volume of fill will be 5 ml of a 10% protein solution in a 20-ml vial. The 5 ml will be isotonic and contain 2% glycerine at pH 6.4 to 6.8" (158).

The most commonly employed current method for purification of the immunoglubulins is ammonium sulfate precipitation. This is also the method employed by Wyeth Laboratories for their antivenins. Some laboratories purify their products by pepsin digestion, and a few use both ammonium sulfate precipitation and pepsin digestion (see Antivenin List, Table II). Some countries do not state their purification methods, while two or three apparently continue to use crude horse serum.

The final product is then adjusted to the required standard of potency as laid down by the national authority: made isotonic, a preservative added, filtered, checked for pyrogenicity, and then retested. It is then processed into its sterile final form as a liquid or dried product. In general, each vial must contain an excess of 10% of that considered adequate by the national authority. A vial of Antivenin [Polyvalent] Crotalidae contains sufficient material to neutralize 198 mouse LD_{50}s of C. atrox venom, 1,452 LD_{50}s of C. durissus terrificus venom, 858 LD_{50}s of Bothrops atrox venom.

In most clinical papers the amount of antivenin given by a physician is noted as the number of vials. The amount of antivenin in one vial, however, may differ substantially from that in another, for the amounts are usually, but not always, assayed for their neutralization capacity per lot or batch.

One last comment seems indicated: the shelf-life of the product. There is no question that freeze-dried antivenins under vacuum are the most stable, and the liquid preparations the least stable. Thus, attention must be given to the manufacturer's recommendations concerning the product. In general, antivenins in the liquid form, even when stored properly at 5°C, may need to be discarded after two years if their color has changed or a precipitate has formed. They may, however, retain their potency for many years. I have assayed some liquid antivenins after 4 years, when there was no change in their color or consistency, and found their neutralization capacity about the same as when they were prepared. On the other hand, we have tested a Wyeth Laboratory antivenin for its lethal neutralizing property after 20 years of storage and found it to be unchanged. Another sample, however, kept in a rubber-stoppered bottle and opened five times over a 10-year period showed some decrease in its neutralizing titer, as well as a slight change in its color.

In the United States, the shelf-life of the native antivenins is five years. Properly stored, however, these antivenins

TABLE 2
Antivenins Available for the Treatment of Snake Venom Poisoning

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
North America and Mexico			
Wyeth Laboratories Box 8299 Philadelphia, Pennsylvania U.S.A. 19101	*Crotalus d. terrificus* *Crotalus atrox* *Bothrops atrox* *Crotalus adamanteus*	Antivenin (Crotalidae) Polyvalent	Precipitated with ammonium sulphate, and lyophilized.
	Micrurus f. fulvius	Antivenin (Micrurus fulvius)	
Laboratories "MYN", S.A. Av. Coyoacan 1707 Mexico City 12, D.F., Mexico	*Bothrops atrox asper* *Bothrops nummifer* *Crotalus atrox* *Crotalus nigrescens* *Crotalus d. durissus* *Crotalus d. tzabcan* *Crotalus tigris* *Agkistrodon bilineatus*	Snake Antivenin	Enzyme digested, precipitated with ammonium sulphate, and lyophilized.
Gerencia General de Biologicos y Reactivos M. Escobeda 20, C.P. 11400 Mexico D.F., Mexico	*Bothrops atrox asper* *Crotalus b. basiliscus* *Crotalus d. durissus*	Anti-Crotalus and Anti-Bothrops	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Central America			
University de Costa Rica Cuidad Universitaria Rodrigo Facio San Jose, Costa Rica	*Lachesis muta* *Crotalus d. durissus*	Polyvalent serum	Precipitated with ammonium sulphate. Freeze-dried or liquid.
	Lachesis muta *Bothrops alternatus* *Bothrops asper* *Bothrops jararacussu* *Bothrops jararaca* *Bothrops moojeni* *Bothrops lateralis* *Bothrops cotiara* *Bothrops nasutus* *Bothrops neuwiedi*	Polyvalent	
	Micrurus nigrocinctus *Micrurus fulvius*	Antielapidico	
Laboratory Veterinarios Casilla 5584 Guayaquil, Ecuador	*Bothrops asper* *Bothrops atrox* *Bothrops xanthogrammus*	Antibothropico Polivalente	Precipitated with ammonium sulphate and supplied in liquid form.

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
Instituto Nacional de Salud Ave. Eldorado con Carrera Zona G, Bogota, D.E., Colombia	Bothrops atrox Crotalus d. terrificus	Antiophidico Polivalente	Globulin precipitated with ammonium sulphate.
Laboratorio Behrens Ave. Principal de Chapellin Apartado 62 Caracas, 101 Venezuela	Crotalus d. terrificus	Anticrotalico	Foreign-protein-reduced
	Bothrops atrox Bothrops venezuelae	Antibotropico	
Instituto Nacional de Microbiologia Avdo Velez Sarsfield 563 Buenos Aires, Argentina	Crotalus d. terrificus	Anticrotalus	Purified by enzymatic and differential thermocoagulation techniques.
	Bothrops alternatus Bothrops neuwiedi	Bothrops Bivalente	
	Bothrops alternatus Bothrops jararaca Bothrops jararacussu Bothrops neuwiedi	Tropical Polyvalent	
	Micrurus frontalis Micrurus corallinus	Antimicrurus	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
Ejercito Argentino Campo de Mayo Batallon 601 Pcia de Bueno Aires, Argentina		Antibothrops bivalente Antimicrurus	
Centro de Zooligia Applicada Universidad de Cordoba Pcia de Cordoba, Republica Argentina	 Crotalus durissus terrificus	Antibothrops bivalente Anticrotalus	
Instituto Butantan Ciaxa Postal 65, São Paulo, Brasil	Crotalus d. terrificus Lachesis muta Bothrops jararaca Bothrops moojeni Bothrops cotiara Bothrops alternatus Bothrops jararacussu Bothrops neuwiedi	Anticrotalico Antilaquetico Antibothropico 	Pepsin-digested, and ammonium sulfate precipitation

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
	Crotalus d. terrificus Bothrops jararaca Bothrops moojeni Bothrops cotiara Bothrops alternatus Bothrops jararacussu Bothrops neuwiedi	Antiophidico Polivalente	
Syntex do Brasia S.A. Rua Maria Candida 1813 São Paulo, Brasil	Crotalus d. terrificus and Bothrops sp.	Polivalente antiophidic serum	Pepsin digestion, and ammonium sulphate precipitationi. Final solution contains 18% protein.
	Bothrops alternatus Bothrops atrox Bothrops jararaca Bothrops jararacussu Bothrops cotiara	Antibothropic serum	
Instituto Vital Brazil S.A. Caixa Postal 28 Niteroi Rio de Janeiro, Brasil	Bothrops sp.	Soro Antibotropico	
	Crotalus d. terrificus	Soro Anticrotalico	
	Bothrops sp.	Soro Antiofidico Polivalente	
	Crotalus sp.		

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
South America			
Institutos Nacionales de Salud Departamento de Animales Venenosos Calle Capac Yupanqui n 1400 Apartade n 451, Lima, Peru	Bothrops atrox Bothrops bilineatus smaragdinus Bothrops castelnaudi Bothrops brazili Bothrops pictus	Suero antibotropico polivalente	Purified and lyophilized
	Lachesis muta	Suero antilachesico	
	Crotalus d. terrificus	Suero anticrotalico	
Europe			
Institut Pasteur Production 3 Boulevard Raymond-Poincaré 92430, Marnes-la-Coquette, France	Vipera ammodytes Vipera aspis Vipera berus	Ipser Europe	Concentrated and purified to 12-13% protein.
	Bitis arietans Bitis gabonica Echis carinatus Hemachatus haemachatus Naja haje Naja melanoleuca Naja nigricollis Naja nivea	Ipser Afrique	

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
	Vipera ammodytes Vipera lebetina obtusa Vipera palestinae Cerastes cornutus Cerastes vipera Echis carinatus Naja naja Naja haje	Near and Middle East	
	Dendroaspis angusticeps Dendroaspis jamesoni Dendroaspis polylepis Dendroaspis viridis	Dendroaspis	
Institut Mérieux 17 Rue Bourgelat 69002 Lyon, France	Vipera aspis Vipera berus	Sérum anti-venimeux purifié Mérieux	
Laboratoires Lelong 45200 Amilly, France	Vipera ammodytes Vipera aspis Vipera berus	Sérum anti-venimeux Lelong	

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
Hoechst Aktiengesellschaft Postfach 80 03 20 D-6320 Frankfurt am Main 80 West Germany	Vipera ammodytes Vipera berus	Europe	Prepared by pepsin digestion, and ammonium sulphate precipitation. Final solution contains 16% protein. Supplied in liquid form.
	Bitis arietans Bitis gabonica Bitis carinatus Naja haje Vipera lebetina	North and West Africa	
	Bitis arietans Bitis gabonica Dendroaspis polylepis Naja haje	Central Africa	
	Echis carinatus Naja haje Vipera ammodytes Vipera lebetina	Near and Middle East	
Twyford Pharmaceutical Services Deutschland GmbH, Postfach 21 08 05 D-6700 Ludwigshafen am Rheim West Germany	Agkistrodon rhodostoma	Malayan pit viper snake antivenin	
	Calloselasma		

(Continued)

Table 2 Continued.

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
Istituto Siereoterapico e Vaccinogeno Toscano "Sclavo" Via Fiorentine 1, 53100 Siena, Italy	Vipera ammodytes Vipera aspis Vipera berus Vipera ursinii	Antiviperin	Enzyme refined and supplied in liquid form.
Istituto Sieroterapico Via Darwin 20, Milano, Italy	Vipera ammodytes		Enzyme refined and supplied in liquid form.
Institut Sérothérapique et Vaccinal Suisse Case Postale 2707 3001 Berne, Switzerland	Vipera ammodytes Vipera aspis Vipera berus	Sérum Antivenimeux	
Serotherapeutisches Institut Wien Triester Strasse 50 A-1100 Vienna, Austria	Vipera ammodytes Vipera aspis Vipera berus Vipera lebetina	Schlangengiftserum, "Sero"	
Institute of Immunology Rockefellerova 2 Zagreb, Yugoslavia	Vipera ammodytes	Antiviperinum	Digested with and precipitated with ammonium

Producer or Distributor	Venoms Used in Preparation	Trade or Common Name	Comments
Europe			
Chemapol Foreign Trade Company, Ltd. Kodanska 46, 100 Praha 10, Czechoslovakia	Vipera ammodytes	Antivipera ammodytes	Pepsin digested and precipitated with ammonium sulphate. Supplied in liquid form.
Institute of Epidemiology and Microbiology Sofia, Bulgaria	Vipera ammodytes (This institute also prepares the same serum in crude form for Albania.)	Monovalent	Ammonium sulphate precipitation.
Ministry of Public Health 101 431, GSP 4 Moscow K-51, U.S.S.R.	Echis carinatus Naja naja Vipera lebetina	Polyvalent	No confirmation indicating product or processing.
	Naja naja oxiana	Anti-Naja	
	Vipera lebetina	Anti-Vipera	
Africa			
Institut Pasteur Rue de Docteur Lavéran Alger, Algérie	Cerastes cerastes Vipera lebetina	Antivipérin	

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